Global Veterinaria 3 (5): 390-394, 2009
ISSN 1992-6197

Comparative Immunological Studies on Avian Influenza Live Fowl Pox Vector H_5 Subtype and Inactivated Avian Influenza H_5N_1 Vaccines

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Abstract: In a trial to control the wide spread of Highly pathogenic avian influenza (HPAI) H5N1 virus outbreaks among poultry flocks in Egypt, many inactivated oil adjuvant AI virus vaccines were used. All of these vaccines were either low pathogenic H5N2 AI viruses or genetically re-assorted H5N1 AI virus. In the current study a recombinant fowl pox-avian influenza (AI) H5 vaccine (reFP-AIV-H5); expressing the hemagglutinin of the A/turkey/Ireland/1378/83 (H5N8) AI isolate; was evaluated in comparison with the genetically re-assorted inactivated H5N1strain A/Goose/Guandong/1/96 and inactivated H5N2 strainA/CK/Mexico/232/CPA/94 in SPF chickens. The potency of the 3 vaccines using HI test against homologous and heterologous AI antigens were 5, 10.2 and 7.7 log2 HI unites, respectively. While, HI titre against the heterlogous AI local antigen prepared from A/chicken/Egypt/12378 N3-CLEVB/2006/H5N1 strain were 2, 6.4 and 5 log2 HI unites, respectively and 0, 4.6 and 0 log_2, respectively against the heterologous AI local antigen prepared from A/chicken/Egypt/9402 NAMRU3-CLEVB 213/2007/H5N1 strain. On the other hand, the efficacy of the 3 vaccines in SPF chickens was studied. The protection percentages were 40, 90 and 80% against HPAI isolate 2006 and were 0, 31 and 19 against HPAI isolate 2007, respectively. AIV shedding was detected and titrated in both vaccinated and control challenged birds. It was concluded that the reFP-AIV-H5 vaccine is not suitable to be used to protect poultry flocks in Egypt against the circulating AIV either 2006 or 2007 strains.

Key words: Immunological Studies · Avian Influenza · Live Fowl Pox Avian Influenza H5N1

INTRODUCTION

Highly pathogenic avian influenza A virus (HPAIV) of subtype H5N1 caused outbreaks in poultry in many Asian, European and African countries including Egypt. In attempts to control the disease, millions of birds have been destroyed. Despite these efforts, HPAI H5N1 virus has become endemic in several regions in domestic and wild birds [1-3]. This situation represents a constant threat to poultry and wild birds in Egypt and world wide. The imminent danger of introduction of HPAIV into domestic poultry led to implementation of vaccination in an increasing number of countries. However, vaccination as a tool to combat HPAIV is a contentious issue. The most convincing argument against vaccination coverage within poultry flocks in Egypt resulting in endemicity rather than in eradication. Continuous circulation of AI virus in vaccinated birds may then result in antigenic drift as has been reported by Taha *et al.* [4] and Lee *et al.* [5]. However, vaccination may also serve as a tool for reduction of viral load in the environment, thus decreasing the risk of transmission within poultry

The current work was planned to study the efficacy of new recombinant vaccine (re FP-AIV-H5) against challenge with the Egyptian HPAI virus strains in comparison with the currently used inactivated AIV vaccines.

MATERIALS AND METHODS

Birds: One hundred and eight, 3 weeks old SPF chickens, were used.

Cell Culture: Primary chicken embryo fibroblasts (CEF) were prepared from 10 day old SPF chicken embryo; that obtained from Kom Oshiem Farm, Fayoum, Egypt and used for titration of fowl pox virus vaccine (According to OIE Protocol [6].

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Embryonated Chicken Eggs: Five hundred SPF embryonated chicken eggs of 9-11 day old were obtained from Koum Osheim SPF Farm, Fayoum, Egypt. They were used for titration of shedding virus.

Vaccines

Recombinant Avian Influenza Fowl Pox Vaccine: Trovac-AIV H5 that contained hemagglutinin gene of AIV strain A/trk/Ireland/1378/1983 H5N8 cy0150089.

H5N1 Inactivated Vaccine: It was prepared in China from Chinese strain A/Goose/Guandong/1/96.

H5N2 Inactivated Vaccine: It was prepared in Mexico from Mexican strain A/ck/Mexico/232/CPA94.

Challenge AI Viruses

Two HPAI Viruses: They were previously isolated and identified and used in our study for both challenge test and antigen preparation.

- The first isolate (Influenza A virus) (A/chicken/ Egypt/12378 N3-CLEVB/2006 H5N1) of accession No. EF469651.
- The second isolate (Influenza A virus (A/chicken/ Egypt/9402 NAMRU3-CLEVB 213/2007 H5N1) accession No. EU623467.

Positive Antisera:

Two Different Anti H5 Sera Were Used: Each one was homologous to the vaccine of the two inactivated AI virus vaccines under study. The sera were obtained from the two imported vaccine companies.

Antigens

Local Antigens (Heterologous): They were prepared from 2006 and 2007 AIV isolates of accession numbers EF469651 and EU623467 respectively.

Imported Antigens (Homologous): Two AI virus antigens of infect A/Goose/Guandong/1/96 (H5N1) for reassorted H5N1 strain and A/ck/Mexico/ 232/ CPA/94 (H5N2) strain were used as homologous AI antigens.

Detection of the H5 Gene Insert in the Recombinant Fowl Pox Vaccine

DNA Extraction: For detection of the H5 gene insert of the AI virus, the viral DNA of the fowlpox virus was extracted from the infected CEF cells using Dneasy kit (Qiagen Company)following the manufacturer instructions.

Polymerase Chain Reaction (PCR): PCR was conducted on the viral DNA of the recombinant pox virus using primer pair specific for H5 gene of AI virus (Table 1) according to WHO H5 Reference Laboratory Network.

Which was modified from *Yuen et al.* [7]. The PCR reaction mixture of 25 µl was 5 µl of 5 x Promega PCR buffer, 0.5 µl of dNTp mix, 1 µl of $Mgcl_2$, 0.7 µl of the forward primer, 0.7 ul of the reverse primer, 1 ul of Taq polymerase, 5 µl of the viral DNA and 10.9 µl of water. The PCR reaction condition was initial denaturation at 95°C for 5 min, then followed by 40 cycles of denature at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min, with final extension at 72°C for 7 min. T-Gradient thermal cycler of Biometra was used.

Phylogenetic Analysis of the HA Gene Segment: The sequence data of HA genes of the 5 AI virus strains used in our study from published works available in the GenBank were analyzed. The sequencing information was complied with the Seqman program in the Lasergene package (DNASTAR Inc.) and the sequences were compared initially with the MagAlign program of the package with the clustal alignment algorithm. Pairwise sequence alignments were also performed with the clustal alignment algorithm in the MagAlign program to determine sequence similarity.

Safety Test: According to OIE [6], ten susceptible chickens were inoculated with 10 field doses of the vaccine and another 10 chickens were kept as an isolated control group. All groups were kept under observation for one month.

Experimental Design: Eighty eight susceptible SPF chickens were used in this experiment. They were divided into 4 groups:

Table 1: Sequence of primer pair species for H5 gene of AI virus

Primer	Sequence	Fragment length
H5 F	GCC ATT CCA CAA CAT ACA CCC	219 bp
H5 R	CTC CCC TGC TCA TTG CTA TG	

Group (I): Vaccinated with recombinant avian influenza fowl pox vaccine via wing web inoculation according to the manufacturer instructions.

Group (II): Vaccinated with H5N1 inactivated vaccine via S/C route at a dose of 0.5 ml/bird.

Group (III): Vaccinated with H5N2 inactivated vaccine via S/C route at a dose of 0.5 ml/bird.

Group (IV)

Non-Vaccinated Control Group: Each group was divided into two subgroups, the first subgroup in groups I, II, III and IV were challenged intranasally with 10^5 AID_{50}/bird of AI 2006 isolate, while the second subgroups were challenged intranasally 10^5AID_{50}/bird AI2007 isolate. All groups were kept under observation for ten days for any clinical signs or deaths.

Titration of the Shedded AI Virus: Swabs were collected daily from vaccinated and non-vaccinated challenged bird from the 3rd till the 9th day post challenge in 2 ml tryptose phosphate buffer with 5 x 10^3 IU penicillin-G sodium and 5 mg streptomycin per ml. The swabs were stored at – 70°C until titrated by inoculation in 10 day old SPF embryonated chicken eggs and the titre was calculated according to Reed and Muench [8].

Serological Tests

Haemagglutination and Haemagglutination Inhibition Tests: They were used for evaluation of the humoral immune response of the vaccinated chicken groups against AI virus vaccines; according to the OIE Standards [6].

RESULTS

Concerning the reFP-AIV H5 vaccine evaluation results, the most important points were: firstly the reFP-AIV-H5 vaccine proved to be containing H5 gene, as detected by PCR; the reFP-AIV-H5 virus vaccine titre is $10^{4.6}$ $TCID_{50}$/dose.

On comparing the potency of the reFP-AIV-H5 vaccine, in SPF chickens; with that of H5N1 (Reassorted) and H5N2 vaccines the results were 5, 10.2 and 7.7 $\log_2$ HI unites against the homologous AI antigen; 2, 6.4 and 5 $\log_2$ HI unites against the heterologous AI antigen prepared from the A/chicken/Egypt, 12378 N3-CLEVB/2006 H5N1 and 0, 4.6 and 0 $\log_2$ HI unites against the heterologous AI antigen prepared from the A/chicken/Egypt, 9402 MAMRU-CLEVB213/2007H5N1, respectively (Table 2).

Regarding the efficacy of the 3 vaccines in SPF chickens against the local A/chicken/Egypt, 12378 N3 CLEVB/2006 (H5N1) virus strain, the protection

Table 2: Avian influenza haemagglutination inhibition log2 titre using different antigens 3 weeks post vaccination

Type of vaccine	Homologous antigen	CLEVB 2006	CLEVB 2007
Recombinant fowl pox	5.0	2.0	0
H5N1	10.2	6.4	4.6
H5N2	7.7	5.0	0

CLEVB:Central Laboratory for Evaluation of Veterinary Biologics.

Table 3: Challenge test against avian influenza disease

Isolate: 2006						Route: I/N											
						Daily Observation											Protection %
				Challenge		---										Total of	and
Vaccines Barcode	Tag	No.	Signs	dose/bird	Identity	1	2	3	4	5	6	7	8	9	10	deaths	virus titre
Recombinant fowl	Isolator (3)	10	Died	10^5	87 %			2	2	1	1					6/10	40 %
pox vaccine			Diseased														
			Virus Shedding					4/5	4/5	5/5	3/5	1/5					10^4
01120782	Isolator (2)	10	Died		95 %			1								1/10	90 %
H5N1			Diseased						1								
			Virus Shedding					5/5	3/5	1/5	1/5						10^4
0112008	Isolator (1)	10	Died		89 %				1		1					2/10	80 %
H5N2			Diseased					1		1							
			Virus Shedding					3/5	3/5	1/5	1/5						10^4
Control	Isolator (4)	10	Died					4	4	1	1					0/10	0%
			Diseased					2	3	1	1						
			Virus Shedding					5/5	5/5	2/2							10^5

I/N: Intranasal

Table 4: Challenge test against avian influenza disease

Isolate: 213/2007						Route: I/N											
						Daily Observation											
Vaccines Barcode	Tag	No.	Signs	Challenge dose/bird	Identity	1	2	3	4	5	6	7	8	9	10	Total of deaths	Protection % and virus titre
Recombinant fowl	Isolator (3)	9	Died	10^5	85 %		1	2	1	3		1	1			9/9	0%
pox vaccine			Diseased					1				1					
			Virus Shedding				5/5	5/5	2/5	3/5		2/5	1/5				10^4
01120782	Isolator (2)	13	Died		94 %			3	2	1		2		1		9/13	31 %
H5N1			Diseased								2		1				
			Virus Shedding				5/5	3/4	5/5	1/3		2/2					10^4
0112008	Isolator (1)	16	Died		84 %			3		4	2	1	1	2		13/16	19 %
H5N2			Diseased						4				1	3			
			Virus Shedding					3/5	4/4	2/5	0/5						10^4
Control	Isolator (4)	10	Died					9	1							10/10	0
			Diseased					1									
			Virus Shedding				10/10		9/9	1/1							10^5

I/N: Intranasal

percentages were 40, 90%and 80%, respectively. On the other hand, the protection percentages against the local A/chicken/Egypt, 9402 NAMRU3-CLEVB213/2007 (H5N1) virus strain, were 0, 31 and 19, % respectively (Tables 3 and 4).

Regarding the AI virus shedding, there was no significant decrease in the virus shedding between vaccinated and non-vaccinated challenged SPF chicken groups.

DISCUSSION

Inactivated oil adjuvant AI vaccines could be used with restricted biosecurity measures in a comprehensive strategy to control HPAI virus outbreaks in many countries including Egypt. Concerns have been raised about inconsistencies in field protection with quality of some vaccines [9, 10]. Te recombinant vaccines contained the H5 gene of AI were used to control AI in Mexico, USA and other countries [11-13].

In Egypt, the current study is designed to evaluate a reFP-AIV-H5 vaccine in comparison with the already applied inactivated oil adjuvant AI vaccines.

The results of potency test of the reFP-AIV-H5 vaccine against local HPAI H5N1, 2006 antigen is 2 log2 HI unites which is very low and non-protective and was significantly lower than that of either H5N1 (6.4 log2 HI unites) and H5N2 (5 log2 HI unites). On the other hand, the potency of reFP-AIv-H5 against local HPAI H5N1 2007 antigen is 0 log2 HIV unites compared with 4.6 log2 and 0 log2 HI unites for H5N1 and H5N2, respectively. These results are confirmed by results of the efficacy test of the three vaccines in SPF chickens when challenged with either local HPAI H5N1, 2006 strain or the local HPAI H5N1, 2007 Strain, the protection percentages were are 40, 90 and 80%, against 2006 strain and 0, 31 and 19%, against 2007 strains, respectively.

For all the three vaccines, the protection percentages against the A/chicken/Egypt/19402/ NAMRU3-CLEVB213/2007/H5N1 strain are significantly lower than that against A/chicken/Egypt/12378 N3-CLEVB, Abbasia, Cairo/2006/H5N1 strain, this could be due to the mutations that is acquired in the 2007 strain [4] whom recorded that there are 11 points of mutations at the amino acids level in the mutant escape 2007 strain and at highly important sites. All of the mentioned findings lead to lower identity percentage and consequently lower protection percentage which was also recorded in the field [4,14].

The above results indicate that the reFP-AIV-H5 vaccine is not suitable to be used to protect poultry flocks in Egypt against the circulating AIV either 2006 or 2007 strains due to the very low protection percentage; 40% against 2006 strain and 0% against 2007 strain. These results were also confirmed by the lower identity percentage (87 and 85%) when the sequence of the A/Trk/Ireland 11378/83 H5N8 strain is aligned with that of the Egyptian A/chicken/Egypt/12378 N3-CLEVB, Abbasia, Cairo/2006/H5N1 and A/chicken/Egypt/19402 NAMRU3-CLEVB, Abbasia, Cairo 213/2007/H5N1, respectively. On the other hand, the protection percentages of the reFP-AIV-H5 in the vaccinated chickens are lower than that of the studied H5N2 and Reassorted H5N1 vaccines against either 2006 or 2007 strain, respectively.These results are due to the proportionally higher identity between the sequence

A/Goose/Guandong/1/96 of H5N1 vaccine and A/Ck/Mexico/232/CPA94 of H5N2 vaccine when aligned with that of 2006 and 2007 strains. These results were in agreement with that of Taha *et al.* [4].

In conclusion, the HPAI H5N1 virus strains show continued mutations either due to the nature of its genome as RNA type or due to immunopressure so it is highly recommended to update the circulating AIV strains in Egypt in a continous manner to be used as challenge virus in evaluating the AIV vaccines.

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(Received: 03//01/2009; Accepted: 01/02/2009)